Characterization of interaction sites in the *Saccharomyces cerevisiae* ribosomal stalk components.

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Summary

The interactions among the yeast stalk components(P0, P1α, P1□, P2α and P2□) and with EF-2 have been explored using immunoprecipitation, affinity chromatography and the two-hybrid system. No stable association was detected between acidic proteins of the same type. In contrast, P1α and P1□ were found to interact with P2□ and P2α respectively. An interaction of P0 with P1 proteins, but not with P2 proteins, was also detected. This interaction is strongly increased with the P0 carboxyl end, which is able to form a pentameric complex with the four acidic proteins. The P1/P2 binding site has been located between residues 212 and 262 using different C-terminal P0 fragments. Immunoprecipitation shows the association of EF-2 with protein P0. However, the interaction is stronger with the P1/P2 proteins than with P0 in the two-hybrid assay. This interaction improves using the 100-amino-acid-long C-end of P0 and is even higher with the last 50 amino acids. The data indicate a specific association of P1α with P2□ and of P1□ with P2α rather than the dimerization of the acidic proteins found in prokaryotes. In addition, they suggest that stalk assembly begins by the interaction of the P1 proteins with P0. Moreover, as functional interactions of the complete P0 were found to increase using protein fragments, the data suggest that some active sites are exposed in the ribosome as a result of conformational changes that take place during stalk assembly and function.

Introduction

The stalk is an important component of the ribosomal GTPase-associated centre, which is involved in the interaction and function of the cytosolic factors during thetranslation process (Möller and Maassen, 1986; Liljas andGarber, 1995; Stark et al., 1997; Agrawal et al., 1998). The bacterial stalk is formed by a very stable complex of five proteins, one copy of protein L10 and two dimers of the acidic protein L7/L12, which withstands high urea concentrations (Pettersson et al., 1976). This pentameric complex binds to the 23S rRNA GTPase centre through the N-terminal domain of L10 (Bocharov et al., 1998), forming a very flexible structure. In eukaryotes, the stalk is structurally similar (Sanchez-Madrid et al., 1979; MacConnell and Kaplan, 1982; Uchiumi et al., 1986; Lavergne et al., 1987; Bargis-Surgey et al., 1999; Gómez-Lorenzo et al., 2000). The pentamer is formed by protein P0 and a group

of small acidic proteins, and interacts with the equivalent rRNA GTPase, which is functionally interchangeable with the one from bacteria (Musters et al.,1991; Thompson et al., 1993). However, there are experimental data suggesting that the eukaryotic stalk performs additional regulatory functions, which are supported by important structural differences (for a review, see Ballestaand Remacha, 1996). Thus, the polypeptide composition of the eukaryotic stalk is more complex. Protein P0 is larger than its bacterial counterpart, protein L10, because of the presence of a C-terminal extension that closely resembles the acidic proteins' end (Shimmin et al., 1989). In addition, the acidic proteins have evolved in most eukaryotes to form two distinct, but closely related families, P1 and P2, which are completed in plants by a third one, P3 (Szick et al., 1998). Moreover, the number of acidic proteins that form the families varies among species. Frequently, the families have only one member (Woolet al., 1991). However, in *Saccharomyces cerevisiae*, the families are made of two members, P1α/P1 and P2α/P2 (Beltrame and Bianchi, 1990; Newton et al., 1990), whereas several members have been reported in protozoa (Schijman et al., 1995). In higher eukaryotes, P0 forms a pentamer with one dimer of each of the acidic proteins P1 and P2 (Uchiumiet al., 1987). However, in species with two members in the P1 and P2 families (as in *S. cerevisiae*), the pentameric complex seems to be made by P0 and one copy of each of the four acidic proteins (Guarinos et al., 2001). No data are available on the stalk acidic protein composition in species with more than four acidic polypeptides, such as protozoa and plants.

Another striking differentiating feature of the eukaryotic stalk is its structural instability. In contrast to the *Escherichia coli* ribosome, the eukaryotic pentameric complex cannot stand high urea concentrations and is easily disrupted by ethanol/ammonium salt buffers that remove the acidic proteins, but not P0, from the ribosome (Towbin et al., 1982). Moreover, an *in vivo* exchange has been found between the acidic proteins in the ribosome and in the cytoplasmic pool in eukaryotes (Zinker and Warner,1976; Tsurugi and Ogata, 1985; Scharf and Nover, 1987), but not in prokaryotes (Robertson et al., 1977). In addition, although the bacterial stalk has a constant amount of bound acidic protein (Subramanian, 1975), the P1/P2 content of the *S. cerevisiae* particles is related to the metabolic activity of the cell (Saenz-Robles et al., 1990). Altogether, the data indicate that the P0 C-terminal extension can provide the minimal structural element required for stalk function (Remacha et al., 1995; Santos and Ballesta, 1995), whereas the 12 kDa P1 and P2 proteins, which are not essential for ribosome activity, appear to modulate the pattern of proteins translated by the ribosome (Remacha et al., 1995). The existing experimental data are consistent with a role for the eukaryotic stalk in the translocation step of polypeptide synthesis (Sanchez-Madrid et al., 1979; MacConnell and Kaplan, 1982; Lavergne et al., 1987; Gómez-Lorenzo et al., 2000), which is, however, unclear. Thus, although the results of affinity labelling studies suggest a physical interaction between the stalk and elongation factor-2 (EF-2), cryo-electron microscopy (EM) reconstitution studies indicate the existence of a physical gap between the stalk and the 80S ribosome–EF-2 complex (Gómez-Lorenzo et al., 2000). Little is known about the interactions between the different components of the eukaryotic stalk. *In vitro* reconstitution studies using purified rat stalk components have indicated that the highly insoluble P0 became soluble upon binding to the P1/P2 proteins (Uchiumi and Kominami,

1992), and that protein P1 seems to play an important role in this process (Gonzalo et al., 2001). However, available information regarding the interactions between acidic proteins, and of these with P0, is very limited. Similarly, experimental data on the assembly of the eukaryotic stalk are scarce. It is known that the presence of at least one protein from each family, P1 and P2, is required for binding to the ribosome (Remacha et al.,1992), but it is not known whether the acidic proteins bind to P0 independently or as a complex. To gather more information on these questions, a systematic study of the interactions among the proteins that constitute the yeast stalk was undertaken. At the same time, the relationship of the stalk components with the elongation factor EF-2 has also been explored. Different technical approaches have been used, but the most interesting data have been obtained by the two-hybrid technique (Chien et al., 1991), a method that has allowed successful study of protein–protein interactions in the cell environment (Ito et al., 2000; Uetz et al., 2000).

Results

Study of individual interactions between ribosomal stalk components

The protein–protein interactions were initially detected by the two-hybrid system using the agar plate assay, a fast method that provided preliminary qualitative results. Then, in a second step, the selected transformants were evaluated using an ONPG-based □-galactosidase test in cell extracts, an assay appropriate for estimating two-hybrid interactions quantitatively. The constructs in the pGBT9 and pGAD424 vectors with the genes encoding the different proteins used in this study were first used individually to transform *S. cerevisiae* PJ69-6, and their respective background activities have been subtracted from the estimated values in all the following data.

Interactions between 12 kDa P proteins

To test for pairwise interactions between the four 12 kDa acidic proteins, the cells were transformed with two constructs, one in pGBT9 and the other in pGAD424, in all possible combinations of the four 12 kDa acidic protein genes.A low level of enzyme induction was noted in cells transformed with plasmids containing the same gene(Fig. 1A). This low induction indicates that the proteins seem to be unable to form stable dimers in these conditions. Similarly, the different members of the same family do not seem to establish strong interactions between them. Thus, the enzymatic activity detected in cells transformed with the two forms of the same protein (α and □) was also low (Fig. 1B), not supporting the existence of stable associations. A substantially higher □-galactosidase induction was detected when proteins of different types were tested, but only in the case of transformants carrying either pGBT9-1□ and pGAD424-2α or pGBT9-1α and pGAD424-2□ (Fig. 1B). These results support the existence of a preferential association between proteins P1□ and P2α in one side and P1α and P2□ in the other. To confirm these preferential interactions, affinity chromatography was performed using ribosomes carrying either P2α or P2□ proteins tagged with a His6 tail at the C-terminal. The tagged ribosomes were bound to Ni^{2+}columns,

disassembled by treating them with RNase A, and the column was washed with buffer until no protein was detected in the collected fractions. Under these conditions, only the ribosomal proteins that are able to interact with the tagged protein will remain in the column. The bound proteins were then eluted with 200 mM imidazole. The different fractions were resolved by SDS-PAGE, and the proteins were identified by Western blotting using specific antibodies (Fig. 2). As expected, proteins P2α-His and P2□-His were eluted from their respective columns (Fig. 2A and B, columns labelled 2). In addition, protein P1□ was mainly present in the fractions eluted from the P2α-His column (Fig. 2A) and protein P1α in the fractions from the P2□ column (Fig. 2B).α□

P0 interacts specifically with the members of the P1 family

It is well known that, in the ribosome, protein P0 is associated with the four 12 kDa proteins to form the pentameric stalk complex; however, it is not known whether the five components make direct physical contact. As a way of exploring the architecture of the complex, the interaction of P0 with each of the acidic proteins was tested. The P0 constructs were co-transformed with each of the four 12 kDa protein plasmids, and the induced activity was estimated in the extracts (Fig. 3). A clear □-galactosidase induction was detected only when the P0 constructs were expressed together with either P1α or P1□. It seems that the interaction of P0 with the 12 kDa acidic proteins followed their division along the family line, as P0 interacted significantly with both P1 proteins but not with the P2 family.

Location of the acidic protein-interacting region in protein P0

It has been reported previously that the acidic protein binding site is located at the carboxyl domain of proteinP0 (Santos and Ballesta, 1995). To identify more accurately the region interacting with the 12 kDa proteins, con-structs carrying two fragments of the P0 C-terminal were prepared. One, $P0_{100}$, contained the 100 C-terminal amino acids (212–312), and the second, $P0_{50}$, included the last 52 amino acids (262–312). They were tested with each of the 12 kDa protein plasmids (Fig. 4).

The $P0_{100}$ construct interacts with both P1 proteins much more strongly than the intact P0 (Fig. 4), inducing a surprisingly high □-galactosidase activity at the level of 300–400 units. A substantial activity induction is not found in the case of $P0_{50}$, indicating that the interaction is lost upon removal of the region comprising amino acids 212–262. A weaker but still strong interaction was detected between the P_{100} construct and P2α, only when the latter was cloned in the pGAD42 vector. However, no activity was detected with the P2□ construct.The strong interaction capacity of the last 100 amino acids of P0, which takes place mainly with the P1 proteins, supports the data obtained with the whole protein, but raises the question whether this fragment is able to form a complex with the four acidic proteins. To test this point, the $P0_{100}$ fragment, tagged with a His6 tail at the amino end, was bound to a Ni^{2+} column and treated with an acidic protein extract. After washing with buffer until no protein was detected in the fractions (Fig. 5, column 1), the column was eluted with 200 mM imidazole. The eluted proteins were resolved by SDS-PAGE and identified with the specific

antibodies (Fig. 5). The four 12 kDa proteins were detected in the fractions together with $P0_{100}$-His. Although the different affinity of each antibody for the corresponding protein does not allow us to draw a conclusion about the relative amount of each bound protein, the results strongly support the capacity of this fragment to form a pentameric protein complex. As expected, no protein was present in the imidazole-eluted fraction of a control column lacking bound $P0_{100}$-His, which was treated with the acidic protein preparation and processed in a similar way to that carrying the P0 fragment (Fig. 5, column 3).

Interaction with the elongation factor EF-2

The ribosomal stalk is directly involved in the protein synthesis translocation step, although the role of each stalk component in the EF-2-dependent function is unknown. To explore the interactions between the different stalk components and EF-2, immunoprecipitation and the two-hybrid methods were used. Protein P0 and EF-2 were labelled separately with [35S]-methionine by in vitro synthesis in a reticulocyte lysate. The level of expression of both proteins in this system is quite dissimilar, probably because of their different sizes and the sensitivity of the factor to proteolysis (data not shown). The samples were mixed, incubated with monoclonal antibody 3BH5, an antibody specific to the carboxyl-terminus of P0, and immunoprecipitated by protein G-sepharose. A radioactive EF-2 band was found associated with the precipitated P0 (Fig. 6A, lane 4). When the antibody was omitted as a control, no precipitated radioactive bands were detected (Fig. 6A, lane 3). As expected, protein P0 alone was precipitated by the antibody (Fig. 6A, lane 1). A weak background band was found when the EF-2 sample was treated with the antibody (Fig. 6A, lane 2). A densitometric estimation of the bands indicates that the amount of EF-2 in lane 4 is about 20-fold higher than that in lane 2, confirming the specificity of the co-immunoprecipitation of EF-2 with P0. The background band could result from an association of EF-2 with the endogenous P0 in the reticulocyte lysate, although alternative interpretations are possible, such as physical trapping, unspecific binding to sepharose, etc. Similar results have been obtained using a rabbit anti-P0 serum (data not shown). To analyse these interactions in more detail, the corresponding two-hybrid *S. cerevisiae* EF-2 plasmids were tested with each of the stalk protein constructs. Reproducible weak interactions were detected with all the stalk proteins (Fig. 7). The EF-2 interaction increased when the assay was performed using the last 100 amino acids of P0 ($P0_{100}$), and it was even stronger with the last 50 residues of the stalk protein ($P0_{50}$). A similar strong stimulation was detected using the last 50 amino acids of both P1 proteins. The equivalent constructs of proteins P2 were not available for testing.

Level of expression of the different P0 fragments in the two-hybrid system

The magnitude of LacZ induction in the two-hybrid system is determined by the level of interaction of the proteins fused to the two Gal4 promoter fragments. It is, however, possible that the amount of □-galactosidase activity is also affected by the different expression of the fused proteins. To exclude this possibility in the case of the different P0 constructs, P0, P_{100} and P_{50}, the amount of fused protein present in the cell was estimated by Western blotting

using antibodies specific to the Gal4p-activating domain (Fig. 8). The results show that the fusion protein containing the whole P0, which shows some degradation products, is in fact present in a higher concentration than the P_{100} fragment, which is found in roughly the same proportion as the P_{50} fragment. The amount of ribosomal protein L12 is used as a reference for the amount of loaded extract. These results therefore support the view that the differences found in the extent of □-galactosidase induction result from an effect at the level of protein interactions rather than protein expression.

Discussion

The capacity of the two-hybrid systems to screen for and detect new structural and functional protein–protein interactions has been confirmed repeatedly (Ito et al., 2000;2001; Uetz et al., 2000). Moreover, relevant data have been obtained in the study of specific interactions among components of cellular multimeric systems (Finley and Brent, 1994; Bartel et al., 1996; Fromont-Racine et al.,1997; Flores et al., 1999). In most cases, however, the reported two-hybrid data are based on the qualitative properties of yeast colonies (blue colour, growth, etc.), which makes it difficult to estimate the strength of the detected protein interactions quantitatively. Moreover, the system is susceptible to errors as it is easily checked by comparing the data from different reported systematic screenings. In this report, the data are based on quantitative biochemical tests of the induced □-galactosidase activity, which yields a more reliable and accurate estimation of the interactions. It is generally accepted that the bacterial acidic proteins form stable dimers in the ribosome as well as in solution (for a review, see Gudkov, 1997). Previous results using purified proteins have indicated that, under the appropriate conditions, the purified P2 proteins from *Artemia salina* (van Agthoven et al., 1978; Uchiumi et al., 1987) and *S. cerevisiae* (Zurdo et al., 1997) were also able to form dimers and higher associations. This aggregation capability, however, seems to be much more reduced in the case of yeast P1 proteins (Juan-Vidales et al., 1984; Zurdo et al., 2000a). Altogether, these results have led to the assumption that the acidic stalk proteins are also present as dimers in the stalk of eukaryotic ribosomes. Experimental data have strongly suggested that this is not the case in the yeast ribosome (Guarinos et al., 2001), and the two-hybrid as well as the affinity chromatography results reported here clearly support this conclusion. All the data indicate that the yeast acidic proteins of the same family have very little tendency to form either homo- (α/α, □/□) or heterodimers (α/□) in the cell. These facts therefore discount the possibility that the structure of the stalk in *S. cerevisiae* is similar to the bacterial one, in which the formation of L7/L12 dimers is an essential element (Gudkov, 1997). The existence of different structures is also supported by the strong physical interaction established between proteins from different families. This conclusion is supported by the results showing a specific association between proteins P1□ and P2α in one side and P1α and P2□ in the other. These interactions may explain several previous observations: the preferential loss of P1□ and P2α when the yeast P0 is replaced by other eukaryotic homologues (Rodriguez-Gabriel et al.,2000), and the specific release of P1a and P2□ upon elimination of protein L12 (Briones et al., 1998). The existence of these preferential associations has also been reported in the cell cytoplasm (Nusspaumer et al., 2000). Some interesting conclusions can also be drawn concerning the stalk assembly process. The

results show that the complete protein P0 is able to associate directly and form a complex with the 12 kDa P1 proteins, but not with the P2 proteins. This is in agreement with the previously reported capacity of protein P1α, but not P2□, to bind to ribosomes lacking acidic proteins in reconstitution experiments (Zurdo et al., 2000b) and with the role of P1 in the in vitro formation of the rat pentameric complex (Gonzalo et al., 2001). Moreover, it was also found that the P2 proteins do not bind to the ribosome and, instead, accumulate free in the cytoplasm in *S. cerevisiae* mutants lacking both P1 proteins (Remacha et al., 1992). These data, together with the presence of P1–P2 heterodimers in the cytoplasm (Nusspaumer et al., 2000), support the view that, in contrast to bacteria, the assembly of the yeast stalk takes place by the interaction with P0 of the P1α–P2□ and P1□–P2α pairs. Furthermore, the interaction of these heterodimers seems to take place through the P1 component, as P2 proteins apparently display a lower affinity for P0. Altogether, the available data support the idea that the *S. cerevisiae* stalk structure is made of one acidic protein of each type, which interacts with P0 forming anon-symmetrical pentameric complex (P1α/P2□)–P0–(P1□-P2α), in contrast to the 'classical' (P1)2–P0–(P2)2 pentamer, apparently present in eukaryotic organisms with only one acidic protein of each type (Uchiumi et al., 1987). However, considering the S. cerevisiae data, it would be convenient to explore the possibility that the 'classical' model is also less symmetrical than expected, with existing(P1/P2)2 heterodimers rather than (P1)2 and (P2)2 dimers. In fact, the initial interaction of P1 in the rat ribosomal stalk, commented upon previously (Gonzalo et al., 2001), suggests that a similar assembly process might take place in mammals and is compatible with this possibility. Recent reports support this model in higher eukaryotes (Tchorzewski et al., 2000; Shimizu et al., 2002).

The two-hybrid data have enabled us to define more precisely the P0 domain involved in the recognition of the acidic proteins. The dramatic increase in its affinity for the P1 proteins displayed by the P0 C100-terminal fragment, comprising residues 212–312, indicates that the binding site should be located in this region, which must be partially hidden in the whole P0. Interestingly enough, the P_{100} fragment is also able to interact with the P2 proteins (Figs 5 and 6), suggesting the existence of a binding site for this protein type in P0, which seems to be masked in the whole protein. Therefore, the P_{100} fragment can interact with the four acidic proteins simultaneously, underlining the relevance of this part of the protein in the assembly of the pentameric stalk complex. Moreover, as the last 50 amino acids in $P0_{50}$ do not have any interacting capacity, the sequence between residues 212 and 262 must play a critical role in the acidic protein binding site. The most relevant feature of this sequence, which contains two possible a-helices, is a high proportion of hydrophobic residues (Fig. 9). This fact suggests that hydrophobic interactions probably play an important role in the binding of the P1/P2 proteins. Also, the presence of four leucine residues at adequate distances might indicate the participation of a leucine zipper in the binding process. However, the existence of a proline in the middle of the sequence, which may deform the structure, notably reduces this possibility. The role of these structural features in stalk assembly is presently being studied. The data available are therefore compatible with the capacity of protein P0 for binding P1 and P2 (see Supplementary material), although the P2 sites are apparently hidden in the free protein. It is possible that, upon interaction with P1, P0 undergoes a conformational change that exposes the acidic protein binding sites fully

allowing the interaction of the P2 proteins. The dramatic increase in the affinity of the P0 carboxyl domain for P1 also supports this change and suggests that the binding site for these proteins is not totally exposed in the free P0 either and is also affected by the protein conformational modifications. These modifications could be induced by binding to the rRNA or by the action of other effectors. In connection with the change of P0 affinity for P1 and P2, it has to be in kept in mind that, in contrast to the bacterial systems, the eukaryotic stalk is highly dynamic, and an exchange of the acidic proteins has been reported to take place during protein synthesis (Zinker and Warner, 1976; Tsurugi and Ogata, 1985). This process also implies that changes in the affinity of P0 for P1 and P2 must be triggered by still-unknown mechanisms. An interaction of rat P1/P2 proteins with EF-2 has been reported in vitro using surface plasmon resonance (Bargis-Surgey et al., 1999). Our results extend this observation, showing a clear co-immunoprecipitation of the factor and protein P0 by an anti ribosomal stalk-specific antibody. The results of the two-hybrid tests indicate that the interaction between EF-2 and the stalk proteins is notably increased when only the last 50 amino acids of the stalk components are used. These results strongly suggest the involvement of the highly conserved C-terminal sequence EEESDDDMGFGLFD, the only common sequence in all the stalk proteins, in the interaction with the factor, as was previously proposed in a stalk model based on deletion studies (Santos and Ballesta,1995). This conclusion is, nevertheless, in apparent disagreement with a recent three-dimensional cryo-EM reconstruction of a yeast 80S ribosome–EF-2 complex, which showed an extended stalk with the tip, where the protein's C-terminal is supposed to be located, far from the bound EF-2 (Gómez-Lorenzo et al., 2000). However, it is possible that the initial contact with EF-2 could take place through the stalk tip and, once the factor binding is stabilized at the stalk base, the initial interaction is released and the stalk returns to its extended conformation. This hypothesis implies that the affinity constant of the EF-2–stalk tip interaction must change during the process as a result of conformational changes in either EF-2 or the stalk components, something that is compatible with our results, as discussed previously.

Experimental procedures

Bacterial and yeast strains

The *E. coli* DH5α strain, used for plasmid manipulations, was grown in LB medium. *S. cerevisiae* Y190 and PJ69-6 were used as reporter host strains for protein interaction studies. *S. cerevisiae* D4-P2α-His and D5-P2α-His were obtained by transforming strains D4 (lacking protein P2a; Remacha et al.,1990) and D5 (lacking protein P2b; Remacha et al., 1990) with pFL39-P2α-His and pFL-P2□-His (see below). Yeasts were grown in either rich YEPD medium or minimal medium supplemented with the appropriate nutritional requirements (Rose et al., 1990).

Cell transformations

Bacterial transformations were performed according to the methods described by Hanahan (1985). Yeast was transformed using lithium acetate as described previously (Hill et al., 1991; Gietz and Woods, 1994). The transformants were selected on SD plates lacking the required amino acids.

Cell fractionation

Exponentially grown *S. cerevisiae* W303 cells, washed with 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 60 mM NH_4Cl and 5 mM 2-mercaptoethanol (buffer 1), were ground with sea sand as reported previously (Sanchez-Madrid et al., 1979). The extracts were centrifuged for 15 min at 3000 g, and the supernatant was centrifuged again at 100 000 g for 2 h yielding a ribosomal pellet and an S-100 fraction. The ribosomes were washed by centrifugation through a discontinuous gradient of 20% and 40% sucrose in 20 mM Tris-HCl (pH 7.4),100 mM $MgCl_2$, 0.5 M NH_4Cl and 5 mM 2-mercaptoetanol and resuspended in buffer 1. The 12 kDa acidic proteins were obtained by washing the ribosomes with 50% ethanol and 0.5 M ammonium chloride as described previously (Sanchez-Madrid et al., 1979). The split protein fractions (SP), containing the acidic ribosomal proteins, were dialysed on 10 mM Hepes-OHK, pH 7.4, 200 mM ammonium acetate, 1 mM magnesium acetate and 0.5 mM phenylmethylsulphonyl fluoride (PMFS). The preparation was then concentrated by filtration through macrosep 3K omega membranes (Pall/Gelman Laboratory) up to a final concentration of around 1 mg ml^{-1}.

Estimation of □-galactosidase activity

In the two-hybrid system, the joining of two Gal4 promoter domains fused to proteins that are able to interact in the cell induces the transcription of the LacZ gene and, consequently, the expression of □-galactosidase activity. The level of induced enzymatic activity is proportional to the interaction capacity of the proteins fused to the Gal4 domains (Chien et al., 1991). To make a biochemical estimation of the expressed enzymatic activity, a number of transformed colonies were mixed and grown in liquid medium up to stationary phase. Cells, collected by centrifugation, were resuspended in 200 ml of 100 mM potassium phosphate, pH 7.0, and broken in a Fast-Prep equipment (Bio-Rad). The samples were centrifuged in a bench top centrifuge at 12.000 r.p.m. for 15 min, and the amount of protein in the supernatant was estimated according to the method of Bradford (1976). Aliquots of the supernatant (10–100 ml depending on the protein concentration) were mixed with 0.9 ml of buffer Z (65 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 40 mM 2-mercaptoethanol, pH 7.0) and 200 ml of ONPG (4 mg ml^{-1} in buffer Z, pH 7.0). Samples were incubated at 28°C until a strong yellow colour appeared in a positive control. The reaction was stopped with 500 ml of 1.0 M Na_2CO_3, and the A_{420} of the samples was estimated. The enzymatic activity was expressed according to the method described by Rose et al. (1990) and represents the average of four to six experiments performed with different cell extracts from different cell transformations.

Plasmid constructions

The genes encoding proteins YP1α, YP1□, YP2α, YP2□ and P0 were cloned in the *Eco*RI and either *Bam*HI (P1/P2 constructs) or *Pst*I (P0 constructs) sites of the pGBT9 and pGAD424 vectors (Clontech). Previously, the respective restriction sites were introduced at the appropriate gene positions by polymerase chain reaction (PCR) using the previously cloned genes as template. Two C-terminal fragments of protein P0 from positions 213–312 and 261–312 were also cloned in the vectors using a similar strategy. The YP1□, YP2α and YP2□ genes, initially called L44', L44 and L45, were previously cloned in our laboratory (Remacha et al.,1988). The YP1α and P0 genes (Newton et al., 1990) were kindly provided by Dr P. P. Dennis (Vancouver University). The plasmids containing elongation factor EF-2 were prepared similarly from the *S. cerevisiae* EFT2 gene by Dr M. Gómez-Lorenzo at the Glaxo-Wellcome Research Centre (Madrid). The open reading frames (ORFs) of P0, $P0_{100}$ and EF2 were cloned into the *Bam*HI and *Eco*RI sites of the pRSET-A vector (Kroll et al., 1993) to obtain His-tagged proteins under the control of the T7 polymerase promoter for *in vitro* transcription. Tagging of P2α and P2□ was with a His6 tail was carried out using appropriate custom-made oligonucleotides containing the tag, which were fused to the 3' end of the gene coding region by PCR. The tagged genes were afterwards cloned into the pFL39 vector (Bonneaud et al., 1991) obtaining plasmids pFL39-P2α-His and pFL39-P2□-His respectively. All the constructs were confirmed by sequencing. DNA sequencing was performed in an automatic DNA sequencer at the Centro de Biología Molecular DNA sequencing service. In some cases, the correctness of the constructs was confirmed by Western tests of total extracts of the transformed cells, using antibodies specific to the stalk proteins (Vilella et al., 1991).

Protein expression and purification in a prokaryotic expression system

Histidine-tagged $P0_{100}$ protein expression was induced in the *E. coli* strain BL21(DE3)pLysS (Studier et al., 1990) (A_{600} = 0.4–0.8) at 37°C for 1–2 h with 1 mM IPTG. The cells were collected and resuspended in a buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% TritonX-100, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mg ml^{-1} leupeptin, 7 mg ml^{-1} pepstatin,10 mg ml^{-1} aprotinin and 2.5 mg ml^{-1} DNase, RNase. The cell wall was disrupted by repeated freeze–thaw cycles, and the lysate was centrifuged at 12 000 g for 10 min at 4∞C. The fusion proteins obtained were affinity purified on Ni^{2+}His-bind resin (Clontech) according to the manufacturer's instructions.

Analysis of protein interactions by affinity chromatography

A suspension in 50 mM NaH_2PO_4, pH 8.0, 100 mM KCl of resin (100 ml) containing bound $P0_{100}$-His6 protein was treated with 5 mg of an acidic protein preparation (fraction SP) for 3 h at room temperature. The resin was then packed in a column collecting the flowthrough fraction, which was again passed through the column several times. The column was then washed three times with 2.5 volumes of the same phosphate buffer and, finally, the bound proteins were eluted with 2.5 volumes of 50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl and 200

mM imidazole. The proteins in the different collected fractions were analysed by SDS-PAGE and detected by Western blotting using specific antibodies. Ribosomes (3 mg) carrying proteins P2α (A) and P2□ (B) tagged at the C-terminal with a His6 tail were bound in 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$,10 mM imidazole to Ni^{2+} columns for 30 min at 4°C. Afterwards, RNase A (20 mg ml^{-1}) was added, and the resin was incubated for 10 min at room temperature to disassemble the particles. Non-bound proteins were removed by extensive washing with the same buffer and, finally, the bound proteins were eluted with 200 mM imidazole. Fractions were analysed as described above.

In vitro binding assays

35S-labelled proteins were made by TNT reticulocyte lysate system (Promega) from pRSET-H6T-EF2 and pRSET-H6T-P0. *In vitro* transcription and translation assays (50 ml) were carried out in the presence of 1 mg of the plasmid and 6 ml of [35S]-Met (5 mCi ml^{-1}; Amersham) at 30°C for 60 min. To immunoprecipitate the *in vitro*-synthesized proteins, 5 ml of the transcription/translation mixture in buffer A was incubated with 5 ml of undiluted anti-P0 IgG previously bound to the protein G-Sepharose for 60 min at 4°C, and the immunoprecipitates were collected and washed as described before. The products were resolved by SDS-PAGE (10% acrylamide) and detected by autoradiography. To study the interaction of P0 and EF-2, 5 ml of each TNT mixture was incubated together for 60 min at 30°C and immunoprecipitated with the anti-P0 IgG as before.

Electrophoretic methods

Total-cell extracts were analysed by SDS-PAGE using either 10% or 15% acrylamide gels. Proteins were detected by Coomassie blue staining, autoradiography or Western blotting using antibodies specific to the different stalk proteins (Vilella et al., 1991). Acidic ribosomal proteins were resolved by isoelectrofocusing (Zambrano et al.,1997).

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Supplementary material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/mole/mole3179/mmi3179sm.htm.

Method of immunoprecipitation of stalk components with specific antibodies to the acidic proteins and immunoprecipitation analysis.

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Figure legends

Fig. 1. Induction of □-galactosidase activity in *S. cerevisiae* PJ69-6 cells transformed with pGBT9 and pGAD424 constructs carrying 12 kDa acidic protein genes. **A.** Both constructs contain the same protein. **B.** Each construct contains a different gene. Estimation of □-galactosidase activity in extracts was carried out as indicated in Experimental procedures. Marks at the top of the bars indicate the standard deviation of the average values.

Fig. 2. Interaction of 12 kDa proteins estimated by affinity chromatography. Ribosomes carrying proteins P2α (**A**) and P2□ (**B**) tagged at the C-terminal with a His6 tail were bound to Ni^{2+} columns and treated with RNase A to disassemble the particles. Non-bound proteins were removed by extensive washing with the same buffer and, finally, the bound proteins were eluted with 200 mM imidazole. The proteins in the last washing fraction (1) and in the imidazole-eluted fraction (2) were analysed by SDS-PAGE and detected by Western blotting using specific antibodies as indicated.

Fig. 3. Interactions of protein P0 with the 12 kDa proteins. ProteinP0 cloned in pGBT9 (empty bars) and pGAD424 (filled bars) was co-transformed with the constructs from the different 12 kDa proteins in the complementary plasmids as indicated. Estimation of activity as in the legend to Fig. 1.

Fig. 4. Interaction of different P0 domains with the different 12 kDa proteins. Constructs containing the C-terminal last 100 amino acids ($P0_{100}$) and last 50 amino acids ($P0_{50}$) of protein P0 were tested with the corresponding constructs of each of the acidic proteins. The empty and filled bars correspond to the first protein in the pair cloned in the pGBT9 and pGAD424 plasmids respectively.

Fig. 5. Capacity of the carboxyl-terminal $P0_{100}$ fragment to form a complex with the four acidic proteins. The $P0_{100}$ fragment was tagged at its amino end with a His6 tail. The tagged protein was bound to a Ni^{2+} column and treated with a solution of the four acidic proteins. As a control, a Ni^{2+} column without any bound protein was treated similarly. The columns were washed with buffer and then eluted with imidazole. The proteins in the different fractions, resolved by SDS-PAGE, were detected by Western blotting using specific antibodies (Abs), either monoclonal 3BH5, which recognizes the C-terminal of all the stalk proteins including P0, or antibodies specific for each acidic protein. No protein was detected by 3BH5 in either

the last washing fraction (1) or the control column lacking a bound $P0_{100}$ fragment (3). Two bands were recognized, however, by the antibodyin the imidazole-eluted fraction (2); the upper one corresponds to the P_{100}-His fragment and the lower one to the P1/P2 proteins. The presence of each of the four acidic proteins in this fraction was confirmed using monoclonal antibodies specific for P1□, P2α and P2□ (Vilella et al., 1991) and a rabbit serum anti-P1α as indicated.

Fig. 6. Co-immunoprecipitation of EF2 and P0. **A.** *In vitro*-translated EF-2 and P0 protein were metabolically labelled with [35S]-methionine/cysteine in a reticulocyte lysate and immunoprecipitated with monoclonal 3BH5 antibody specific to the carboxyl-terminus of P0. The pellet was resolved by SDS-PAGE, and the radioactive bands were detected by autoradiography. The immunoprecipitation reaction was carried out on the P0 protein sample alone (1), on the EF-2 sample alone (2) and in a mixture of both samples either in the absence (3) or in the presence of the antibody (4). **B.** EF-2 carrying a His tag was expressed in BL21 cells and purified by Ni^{2+} affinity chromatography using a Probond column. The purified EF-2-His was checked by SDS-PAGE and detected by Coomassie blue staining (2). An aliquot of the purified factor was incubated with antibody 3BH5, and the immunoprecipitated material was resolved by SDS-PAGE. The presence of EF-2 in the gel was tested by immunoblotting using the antibody to P0 (3). Molecular weight markers (1).

Fig. 7. Interactions between elongation factor EF-2 and the stalk proteins. Elongation factor EF-2 cloned in pGBT9 was tested with the complementary constructs carrying all the stalk proteins as well as the C-terminal fragments of P0, P1α and P1□

.

Fig. 8. Estimation of the amount of the different P0 fragments expressed from the pGAD424 constructs in *S. cerevisiae*. Cells were resuspended directly in the 10% SDS loading buffer (Laemmli, 1970), boiled for 10 min and centrifuged for 15 min at 3000 g to remove the insoluble material. Soluble extracts were resolved by SDS-PAGE, and the fusion proteins were detected by Western blotting with an antibody specific to the corresponding Gal4 fragment.

Fig. 9. Amino acid sequence of the proposed P1/P2 binding region in the P0 protein. Relevant structural features are indicated. Leucine residues involved in a putative leucine zipper are marked in bold. Hydrophobic residues are underlined. Parts of the sequence with a possible a-helix structure are marked.

Figures

Figure 1.

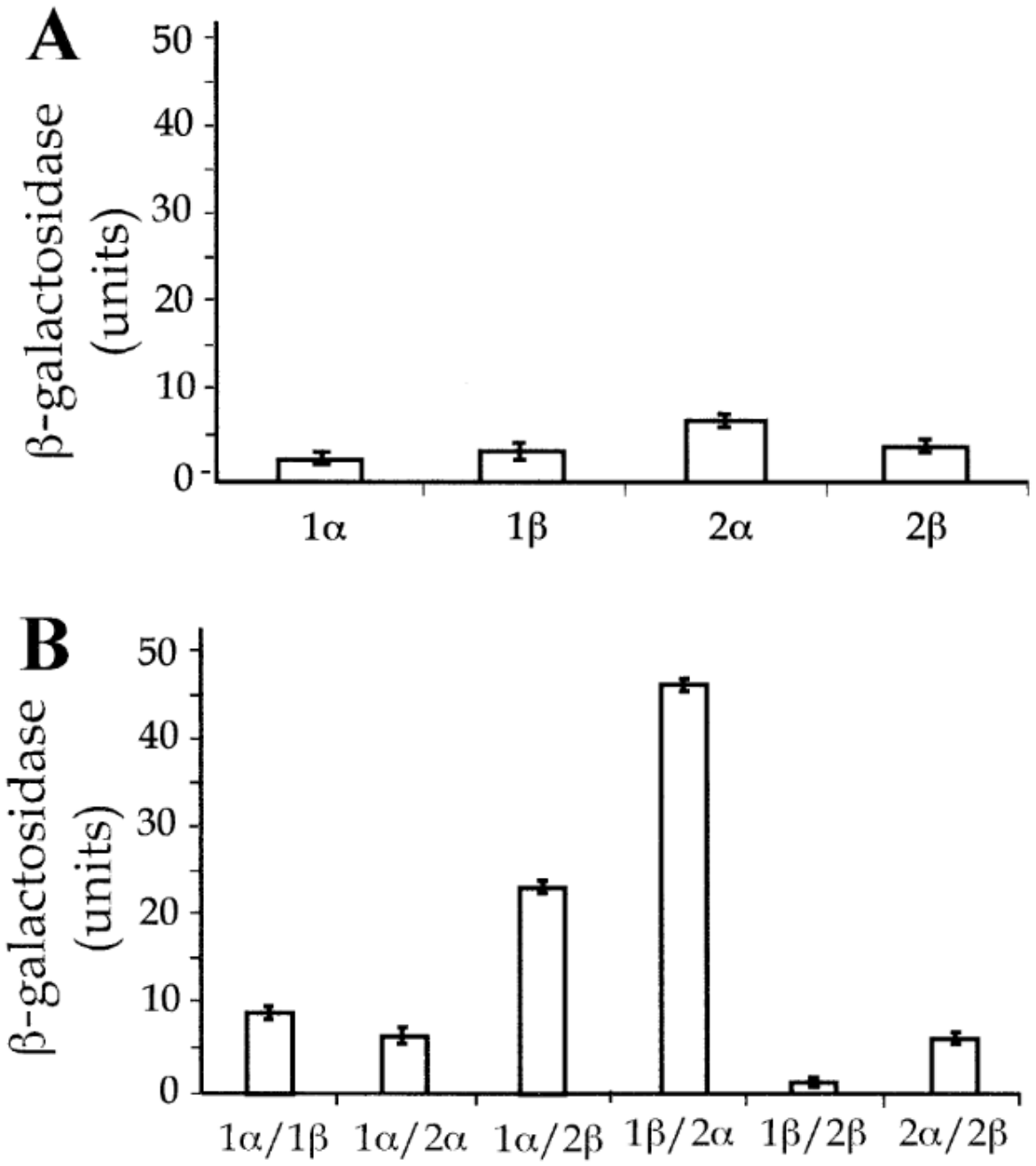
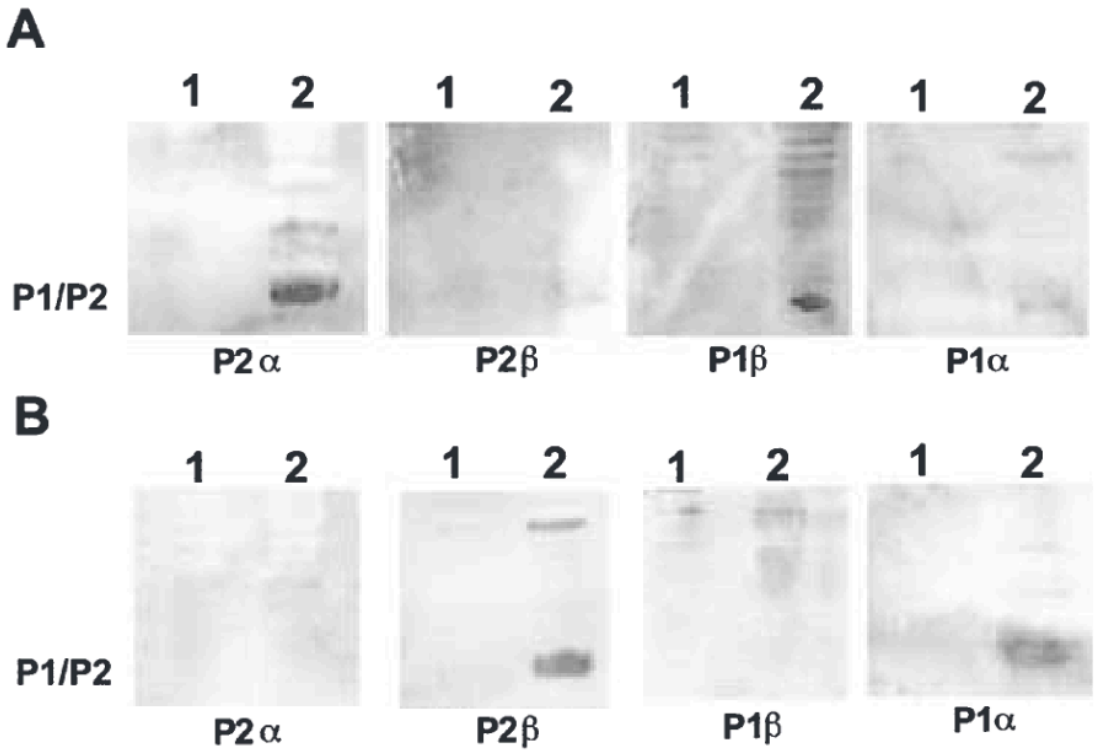


Figure 2



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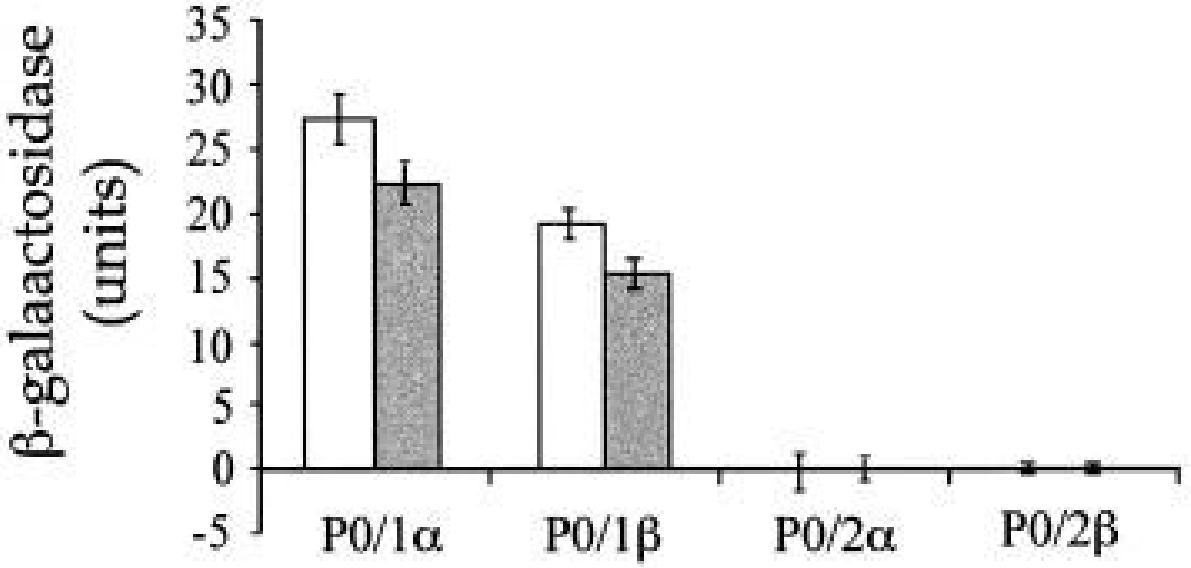

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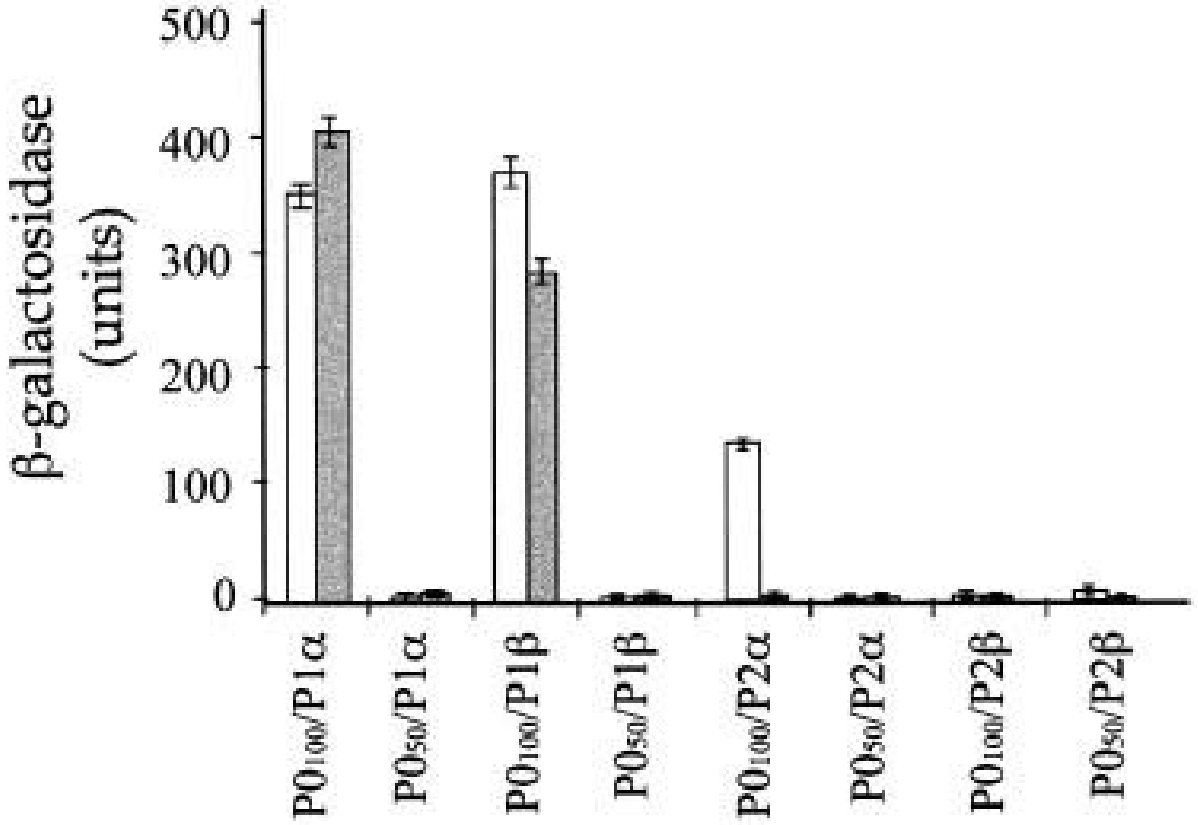

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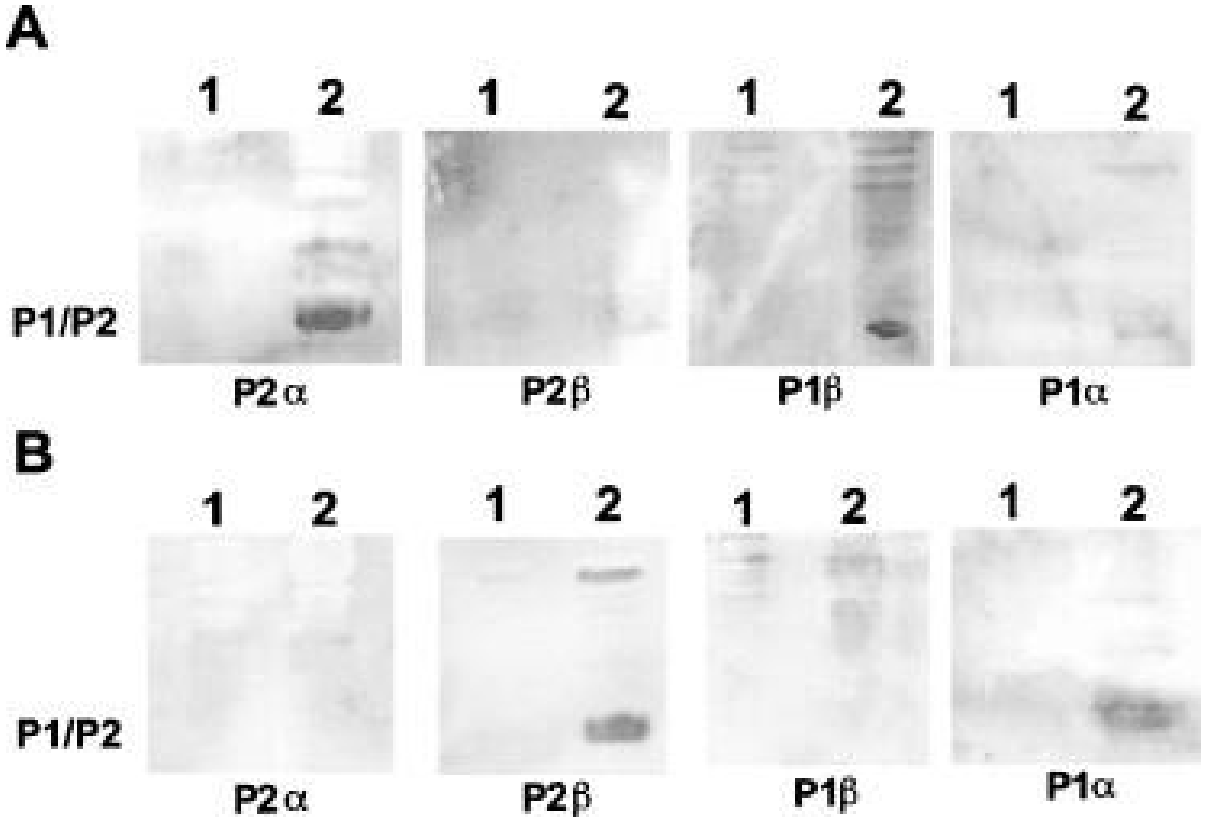


Figure 6.

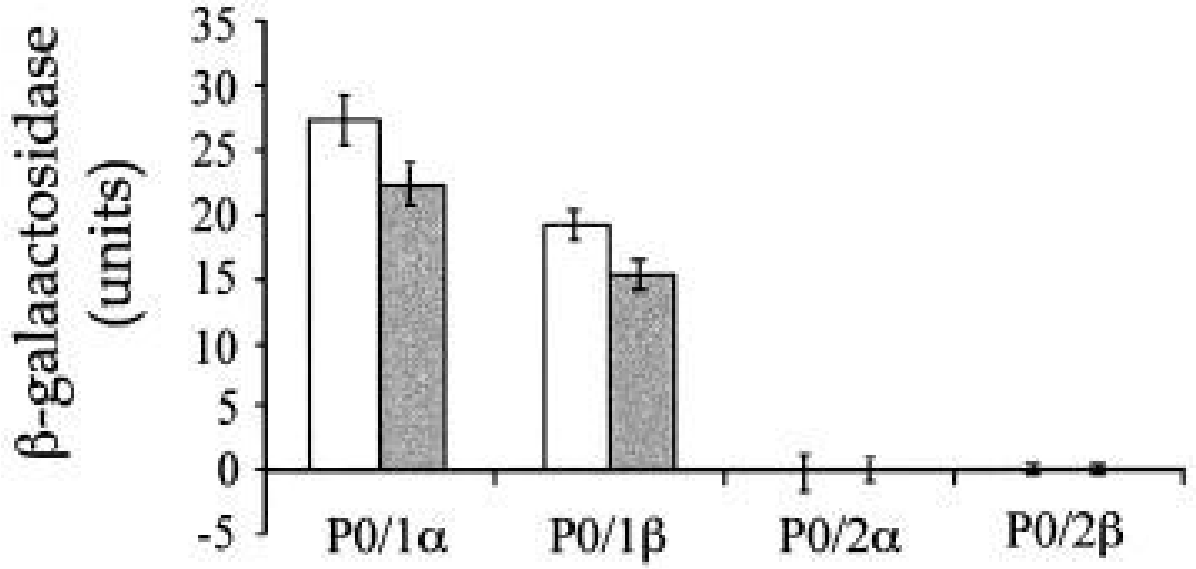


Figure 7.

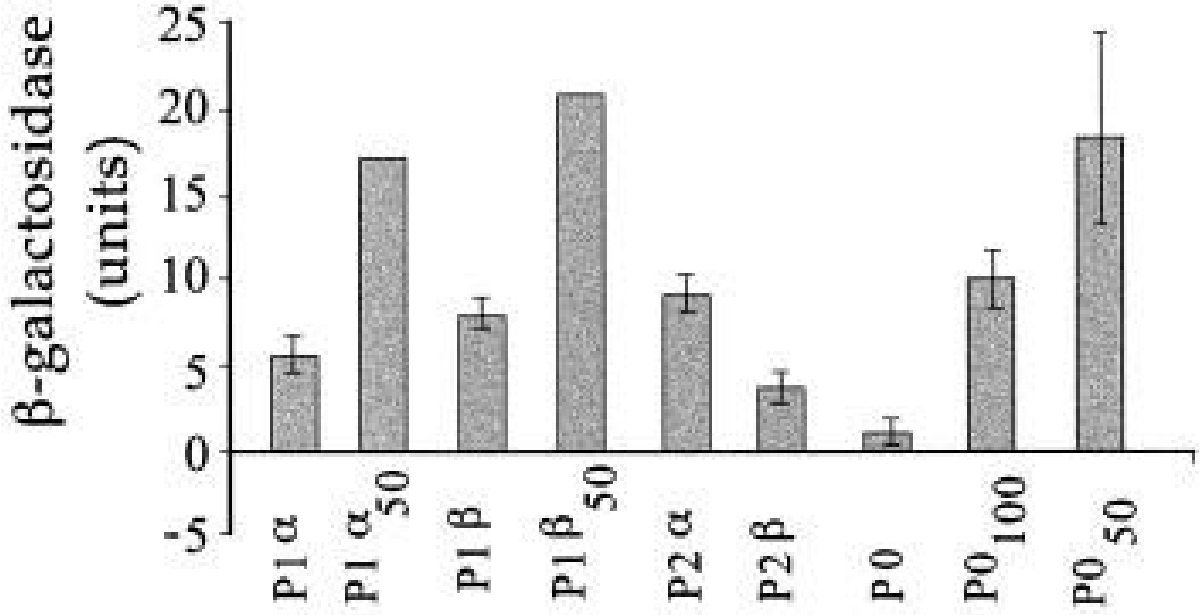


Figure 8.

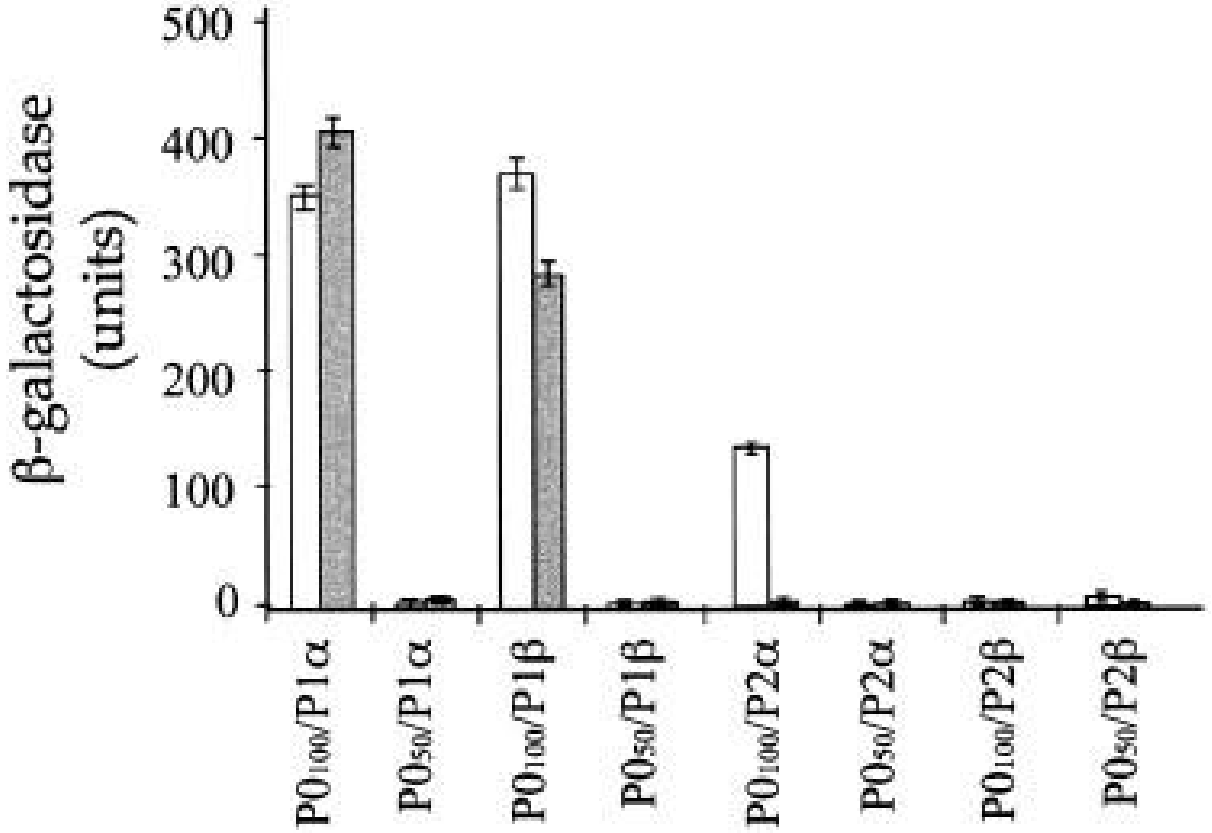
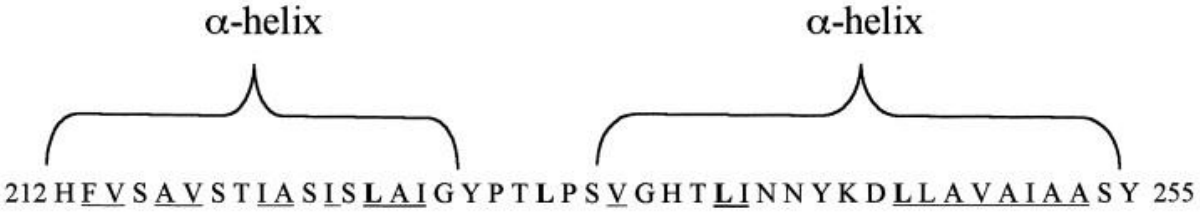


Figure 9.



Supplementary

Immunoprecipitation of stalk components with specific antibodies to the acidic proteins

To study the interactions between the different stalk components immunoprecipitation was tested using specific antibodies to the different yeast stalk proteins previously obtained [Vilella, 1991 #376]. It was found that the weak affinity of the monoclonal antibodies raised against acidic proteins P1□, P2α and P2□ for protein A and protein G (immunoglobulins kappa light chain) precluded their use in immunoprecipitation experiments. However, a polyclonal antibody developed against P1α, which interacts strongly with protein A, allowed carrying out the proposed study.

Ribosomes from *S. cerevisiae* W303, previously disrupted by RNase treatment, were incubated with the anti-P1a polyclonal antibody. The resulting precipitated material was resolved by either IEF or SDS/PAGE and the components analyzed by western blot using the 12 kDa acidic proteins specific antibodies as well as a polyclonal anti-P0. Isoelectrofocusing of the immunoprecipitated samples showed the presence of the four 12 kDa components of the ribosomal stalk (Fig.1 A). The phosphorylated and dephosphorylated forms of the proteins are present, although the ratio of both forms depends on the preparation [Ballesta, 1996 #3181], probably due to phosphatase activity during cell fractionation. In addition, a western blot analysis with the anti-P0 serum of the immunoprecipitate SDS-PAGE gels also revealed the association of P0 with protein P1α (Fig 1B). Protein P0 was not detected in control experiments in which the precipitation was performed with a pre-immune serum, and was drastically reduced when purified P1a protein was added to the immunoprecipitation reaction (Fig 1 C). A similar reduction of P1□, P2α and P2□ was also found using specific monoclonal antibodies (data not shown). The data indicate that after ribosome disruption all the acidic proteins remain interacting with each other and with P0 forming a ribosomal stalk-like complex.

In parallel, immunoprecipitation experiments were also performed to the postribosomal supernatant S100, using the same anti-P1a antibody. The cytosolic acidic proteins also appear to be associated with P1a before their binding to the ribosome, and they are also present in their phosphorylated and unphosphorylated forms (Fig.1A). In this case, protein P0 is not detected.

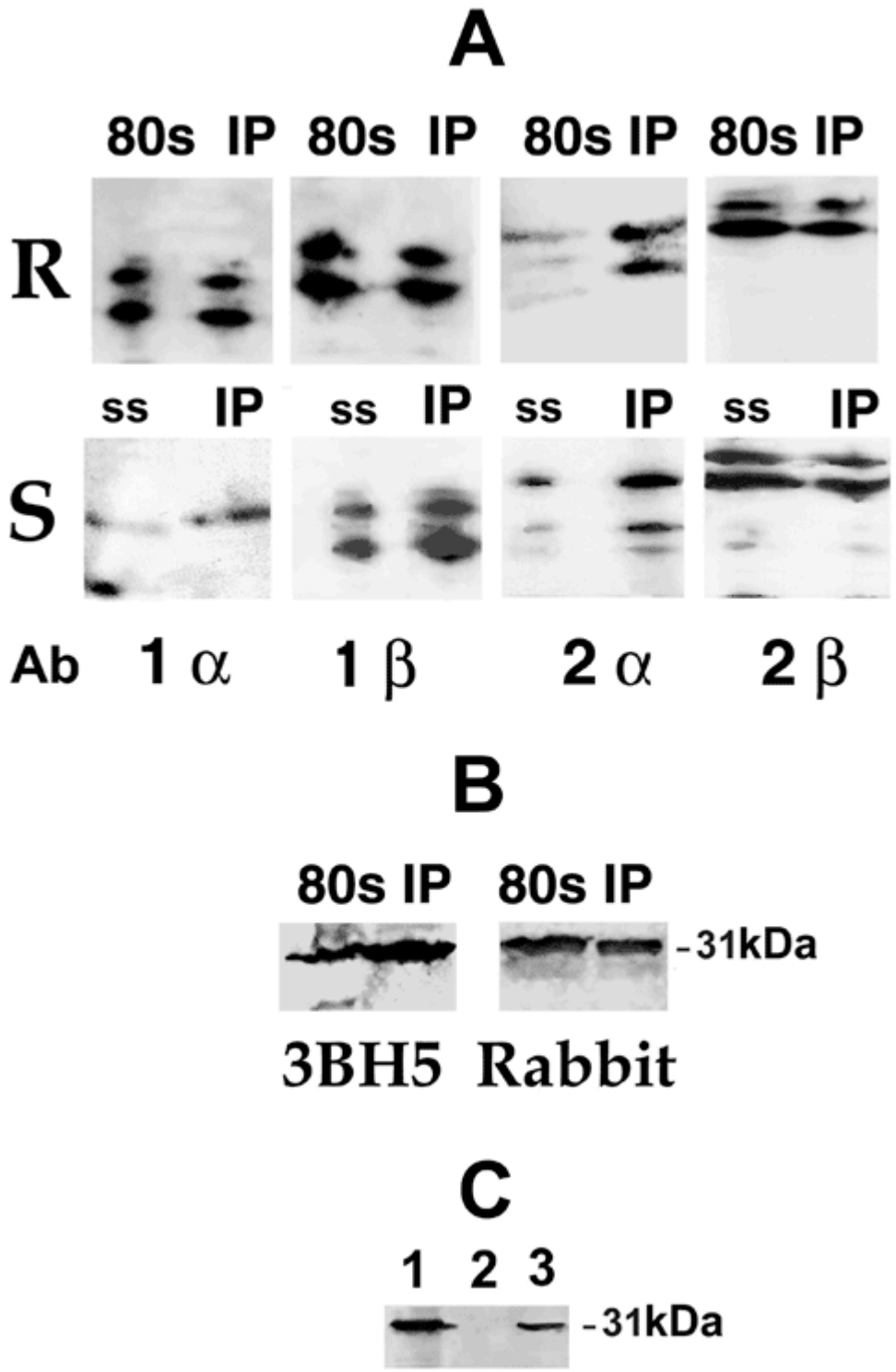
Immunoprecipitation analysis

Ribosomes from *S. cerevisiae* strain W303 obtained as reported [Sanchez-Madrid, 1979 #182] were dissolved in buffer A (50mM Tris-HCl pH 8, 150mM NaCl, 0,5% Triton X 100, 1mM phenylmethylsulfonyl fluoride, 1mM sodium ortovanadate, 10mM sodium fluoride 5mg/ml leupeptin, 7mg/ml pepstatin, 10mg/ml aprotinin) and were incubated with RNase (20mg/ml) for 40min at 4°C. Equal protein concentration (1mg) of ribosomes was immunoprecipitated with the indicated antibodies. After 4h at 4°C immunoprecipitates were mixed with protein G-shepharose for 1h and the immunocomplexes were washed four times

with buffer A and once with the same buffer containing 0.1% SDS. The products were resolved by vertical isoelectrofocusing (pH 2.5 to 5.0) [Zambrano, 1997 #4002] or by SDS/PAGE 15% acrylamide and subjected to western blot analysis.

Fig. S1. Immunoprecipitation of the acidic proteins and P0 with the antibody against P1α. **A.** *S. cerevisiae* ribosomes (R), previously treated by RNase A, and S100 fraction (S) were treated with rabbit antiserum to protein P1α. The samples were incubated for 4 h at 4°C before adding the protein G-Sepharose. After extensive washing, the pellets (IP) were resolved by isoelectrofocusing and transferred to immobilon-P membrane for immunobloting with antibodies (Ab) against the acidic proteins as indicated in the figure. The phosphorylated (lower band) and dephosphorylated form (upper band) of the proteins are detected. Untreated ribosomes (80s) and S100 fraction (ss) were included as a control. **B.** Ribosomes were treated as in (A) and the immunoprecipitate (IP) was resolved by SDS-PAGE and immunoblotted with either a specific rabbit anti-P0 antibody (Rabbit) or with monoclonal antibody 3BH5 against the conserved carboxyl end of the stalk proteins. Untreated ribosomes (80S) were used as a control. **C.** 80S ribosomes, treated as in (A), were immunoprecipitated either with rabbit anti-P1α (1), or with pre-immune serum (2) or with anti-P1α in the presence of purified P1α protein (3). The immunoprecipitated material was resolved by SDS-PAGE and the proteins detected by immunobloting using anti-P0 antibody.

Figure S1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R
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S
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B
80s IP 80s IP
-31kDa
3BH5 Rabbit
C
1 2 3
-31kDa